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(54) **Method for making genetically modified microorganisms.**

(57) A method for making a genetically modified microorganism wherein DNA material, particularly material comprising a *gdh* gene, is transferred from a donor microorganism to a recipient microorganism using a plasmid vector. The invention relates in particular to the use of multicopy plasmids and/or DNA fragments having at their ends target sites for *SalI* restriction enzymes. The method may involve a plurality of steps, transfer of DNA fragments into intermediate microorganisms and use of a plurality of plasmids. Genetically modified microorganisms and a process for the production of single cell protein using the genetically modified microorganisms are also claimed.

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Method for making genetically modified microorganisms

This invention relates to a method for making genetically modified microorganisms, to genetically modified microorganisms and to a process for the production of single cell protain (SCP) using such genetically modified microorganisms.

In our UK Patent No. 1370892 we describe and claim a process for the production of SCP in which bacteria belonging inter alia to the species Methylophilus methylotrophus (formerly named Pseudomonas methylotropha) are cultivated in a medium comprising methanol as a carbon source and appropriate inorganic nutrients. In such a process it is desirable that a high carbon conversion (i.e. conversion of substrate (methanol) carbon to cellular carbon) is achieved. Ammonia is a convenient nitrogen source for such a process.

In the production of SCP by microbiological assimilation of methanol and ammonia, two pathways for ammonia assimilation by microorganisms have been identified, namely

(a) direct (GDH) pathway -

$$HO_2C(CH_2)_2CO\ CO_2H + NH_3 + NAD(P)H$$

alpha keto glutarate

$$\longrightarrow$$
$$\longleftarrow$$

$$HO_2C(CH_2)_2\ \underset{\displaystyle NH_2}{\overset{\displaystyle |}{CH}}\ CO_2H + NAD(P)$$

glutamate

This reaction is catalysed by the enzyme glutamate dehydrogenase

(GDH) and requires one mole of NAD(P)H, equivalent to 3 moles of ATP per mole of ammonia assimilated.

(b) 2-stage (GS-GUS) pathway -

$$HO_2C(CH_2)_2\underset{NH_2}{CH}\,CO_2H + NH_3 + ATP \rightleftharpoons H_2NCO(CH_2)_2\underset{NH_2}{CH}\,CO_2H + ADP + Pi$$

glutamate glutamine

This reaction is catalysed by the enzyme glutamine synthetase (GS).

$$HO_2C(CH_2)_2\,CO\,CO_2H + H_2NCO(CH_2)_2\underset{NH_2}{CH}\,CO_2H + NAD(P)H \rightleftharpoons 2HO_2C(CH_2)_2\underset{NH_2}{CH}\,CO_2H + NAD(P)$$

This reaction is catalysed by the enzyme glutamine glutamate aminotransferase (GUS) (also known as glutamate synthase and as GOGAT). This combination of reactions forming the two-stage pathway requires one mole of ATP for the first stage and the equivalent of 3 moles for the second stage, i.e. a total of 4 moles of ATP, per mole of ammonia assimilated.

The exact mechanism by which ATP is consumed is immaterial. However overall the greater consumption of ATP in the two-stage mechanism results in conversion of more of the methanol to carbon dioxide than in the direct mechanism.

We have found that Methylophilus methylotrophus lacks the GDH enzyme and assimilates ammonia by the two-stage pathway and that this pathway predominates amongst methanol-utilizing bacteria proposed to date for SCP production. In our published UK Patent Specification No. 2003926A we describe an improved process for the production of single cell protein using micro-organisms such as Methylophilus methylotrophus which have been genetically modified by incorporation of the gene specifying ammonia assimilation by the GDH enzyme route. This process gives improved conversion of substrate carbon to cellular carbon.

We have now developed an improved method for introducing genetic material, such as the gene specifying ammonia assimilation by the GDH enzyme route, into a microorganism, thereby producing improved genetically modified strains of e.g. Methylophilus

methylotrophus and an improved process for the production of SCP. In particular our improved genetic modification method provides a means whereby an increased amount of the gene specifying ammonia assimilation by the GDH route can be introduced into e.g. a strain of Methylophilus methylotrophus thereby increasing the amount of the GDH enzyme present in the microorganism and improving the efficiency of the ammonia assimilating pathway established in the microorganism.

Explanation of terms and symbols

In this specification the terms and symbols listed below have the meanings given:-

NAD(P)	-	nicotinamide adenine dinucleotide (phosphate), the co-factor of the GDH or GUS enzyme (and many other enzymes) in its oxidised form;
NAD(P)H	-	nicotinamide adenine dinucleotide (phosphate), the co-factor of the GDH or GUS enzyme (and many other enzymes) in its reduced form;
ATP	-	adenosine triphosphate;
ADP	-	adenosine diphosphate;
DNA	-	deoxyribonucleic acid - genetic material of most organisms;
Pi	-	phosphate ion;
gdh gene	-	the gene specifying glutamate dehydrogenase, the key enzyme for ammonia assimilation by the GDH enzyme route;
glt gene	-	the gene specifying glutamate synthase, the key enzyme for ammonia assimilation by the GUS enzyme route;
glt B	-	the gene specifying glutamate synthase, the key enzyme for ammonia assimilation by the GUS enzyme route, in E. coli;
rif^r	-	rifampicin resistance - other antibiotic resistances are expressed in similar manner;
Tc^s	-	tetracycline sensitivity - other antibiotic sensitivities are expressed in similar manner;

Mu cts - temperature sensitive Mu phage - Mu is a temperate phage;

transposon - a segment of DNA which is transportable from one replicon to another and which can attach itself into a plasmid and frequently confer properties such as antibiotic resistances thereon, example transposon Tn5.

According to the present invention we provide a method for making a genetically modified microorganism by incorporating DNA material comprising a gdh gene into a microorganism deficient in this gene, so that expression of the gdh gene product, i.e. the active GDH enzyme, occurs in the previously gdh gene-deficient microorganism which method comprises the steps of (a) introducing into a first microorganism having the gdh gene a plasmid vector for said gene, and (b) transferring said gene on said vector into a second microorganism. The second microorganism may be the gdh gene-deficient microorganism intended as the ultimate recipient of the DNA material or it may be an intermediate, the DNA material comprising the gdh gene being transferred via one or more intermediate microorganism(s) from the first microorganism to the gdh gene-deficient microorganism.

Further according to the invention we provide a method for making a genetically modified microorganism by transferring DNA material comprising one or more genes from a donor microorganism having said gene or genes to a recipient microorganism deficient therein wherein the DNA material is transferred into the recipient microorganism on a plasmid vector formed from a multicopy plasmid. The method may comprise a plurality of steps involving intermediary microorganisms during transfer of the DNA material from the donor to the recipient microorganism. Preferably the gene transferred is the gdh gene.

Further according to the invention we provide a method for making a genetically modified microorganism by transferring DNA material comprising the gdh gene from a donor microorganism having said gene to a recipient microorganism deficient therein which comprises a step wherein the gdh gene containing DNA material

is transferred on a DNA fragment having at its ends target sites for the SalI restriction enzyme.

The invention further provides new microorganisms produced by the method thereof, variants and mutants of such microorganisms, processes for producing single cell protein and/or amino acids using such microorganisms, protein derived from such microorganisms, dead cells of such microorganisms and feed - or foodstuffs for animal or human consumption containing such dead cells or protein. The invention further provides as new compositions of matter products produced at intermediate stages of the genetic modification method in particular modified R300B, pRP301 and pACYC184 plasmids incorporating the gdh gene.

The invention further and particularly provides the novel microorganism Methylophilus methylotrophus strain NCIB No. 11585 and variants and mutants thereof, processes for the production of single cell protein and/or amino acids using said strain, variants or mutants, dead cells and amino acids produced by such processes, protein derived from said dead cells and feed - or foodstuffs for animal or human consumption containing said dead cells or protein derived therefrom.

Preferably the method of the invention is used to introduce the gdh gene into gram-negative bacteria lacking this gene.

The microorganism Methylophilus methylotrophus strain NCIB No. 11585 was produced by the genetic modification of strain NCIB No. 10515 as described in Example 1 (strain 11585 is Modification 3). A culture of strain NCIB No. 11585 is deposited at the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland, UK. The microbiological characteristics of NCIB No. 10515 and of the species Methylophilus methylotrophus generally are set out in our UK Patent Specification No. 1370892. The microbiological characteristics of strain NCIB No. 11585 are the same as those of strain NCIB No. 10515 except that strain NCIB No. 11585 is resistant to sulphonamide, streptomycin and kanamycin.

Microorganisms which can usefully be modified by the method of the invention to incorporate the gdh gene include the

following bacteria:-

Methylophilus methylotrophus - cultures of which are deposited as follows:-

NCIB 10508 - 10515 and 10592 - 10596, all inclusive;

NRRL B 5352 - B 5364 inclusive;

FRI 1215 - 1227 inclusive;

Pseudomonas methylonica, the characteristics of which are described in UK Specification No. 1451020 and a culture of which is deposited as follows:-

FRI 2247 and 2248

Pseudomonas methanolica - UK Specification No. 1352728 - ATCC 21704;

Pseudomonas sp. - UK Specification No. 1326582 - ATCC 21438 and 21439;

Methylomonas methanolica - UK Specification No. 1420264 - NRRL B 5458;

Pseudomonas utilis - UK Specification No. 1444072 - FRI 1690 and 1691;

Pseudomonas inaudita - UK Specification No. 1444072 - FRI 1693 and 1694;

Methylomonas clara - USP 4166004 - ATCC 31226.

NB: NCIB is the National Collection of Industrial Bacteris, Aberdeen;

NRRL is the Collection maintained by the US Department of Agriculture, Peoria, Illinois;

ATCC is the American Type Culture Collection;

FRI is the Fermentation Research Institute, Japan.

Such microorganisms may be used in processes for the production of SCP wherein, after modification by the method of the invention, they are aerobically cultured in an aqueous medium containing methanol as a source of assimilable carbon, ammonia and/or ammonium ion at a concentration sufficient to maintain the GDH reaction mechanism, and other inorganic nutrients, followed by recovery of SCP from the culture medium.

The conditions of the process for producing SCP are

conveniently as described in UK Specifications Nos. 1370892 and 1451020, except that the total concentration of ammonia and ammonium ion is preferably maintained at above 0.5 mM in zones of maximum methanol concentration. As a result of the decreased consumption of ATP there is less overall conversion of methanol to carbon dioxide and consequently the process is less exothermic and requires less cooling to maintain the temperature at the preferred level in the range 34 - 45°C. For optimum economy the active microorganism predominantly and preferably exclusively metabolises ammonia by the GDH route.

In the method of the invention for introducing a gdh gene into a microorganism deficient in this gene any suitable microorganism may form a source for the gdh gene, gram negative bacteria being preferred. Suitable sources include strains of E. coli, Pseudomonas and Klebsiella, although in general terms there is no a priori reason for selecting any particular gram negative bacterium as the source of the gdh gene. However E. coli is a well-known species whose properties have been widely researched. Hence although a wide variety of microorganisms could serve a sources of the gdh gene it is generally convenient to use E. coli for this purpose. In the particular instance where the gdh gene is to be transferred into a strain of Methylophilus methylotrophus, E. coli is further preferred as a source since we have found that the E. coli gdh gene can provide a functional GDH pathway in Methylophilus methylotrophus without any further manipulation of the gene.

In the method of the invention for the transfer of the gdh gene a fragment of DNA comprising the gdh gene is transferred using one or a succession of plasmid vectors from a microorganism having the gdh gene into a microorganism lacking said gene. In its simplest form the method can consist in introducing into the source microorganism for the gdh gene a plasmid which can act as a vector for the gene, mobilising the gene-containing DNA fragment onto the vector and transferring the gene-carrying vector into the microorganism lacking the gdh gene. However many

variations and elaborations of this simple sequence are possible, one being described in detail in Example 1. For instance the plasmid used to take the gene out of the source microorganism may not be readily transferable into the recipient microorganism and it may therefore be convenient to introduce an extra step wherein the gene is transferred from the original plasmid vector onto another plasmid more readily transferable into the recipient microorganism. Such transfers may be effected by any suitable means, conveniently by use of appropriate restriction enzymes and ligases. In another instance the original plasmid vector may be large and may carry a considerable quantity of extraneous DNA making it difficult to manipulate. In this case it is convenient to transfer the gene-carrying DNA fragment onto a smaller plasmid. A suitable sequence of steps for transferring gdh gene from an E. coli strain into a strain of Methylophilus methylotrophus is described in Example 1. The number and exact nature of the steps required will vary in transfers involving different microorganisms depending upon a number of factors including the source and recipient microorganisms concerned and the availability of suitable plasmids and upon general convenience.

The plasmids used can be derived from any source. Suitable sources include E. coli, Pseudomonas and Klebsiella. Useful plasmids include those of the "P" incompatibility group, for example RP4, which are freely transmissible amongst a variety of gram negative bacteria. Other suitable plasmids for use during the transfer sequence include R300B, pRP301 and pACYC184. Preferably the plasmids are ones which are capable of transfer into the recipient microorganism. To facilitate transfer of the gene, e.g. to improve the utility of a plasmid as a vector or to render it capable of transfer into a recipient microorganism, the properties of a plasmid may be modified by associating with it another DNA fragment or fragments. Examples of such other fragments include temperate phages such as Mu and λ phages and transposons such as Tn 5. Such phages and transposons can play a useful part in the transfer of the gdh gene. Temperature sensitive Mu phage (Mu cts)

is useful in the preparation of the plasmid vector used to take the gdh gene from the source microorganism. When a microorganism containing (Mu cts) is grown at a partially inducing temperature transposition of Mu and adjacent genome DNA occurs without further maturation of phage particles. This transposition then facilit- transfer of fragments of the microorganism's DNA onto a plasmid such as RP4 present in the microorganism leading to a recombinant RP4 containing segments of genome DNA. Temperate phages and transposons can also be used to provide marker antibiotic resistances and target sites for restriction enzymes on otherwise deficient plasmids. For example transposon Tn 5 is very useful as a supplier of target sites for the SalI restriction enzyme to plasmids such as R300B, one such variant of R300B:Tn 5 is pTB70.

Groups of plasmids which are readily transferable into strain NCIB No. 1015 and are thus very useful in the invention are the Inc Q, Inc P and Inc W groups of plasmids. Included in the Inc Q group are plasmids R300B and other plasmids described by PT Barth and N J Grinter (J. Bacteriol., (1974), 120, 618 - 30) and plasmid R1162. Included in the Inc P group are plasmids RP4 (described by N Datta et al, J. Bacteriol., (1971), 108, 1244 - 49), pRP 301 (described by P T Barth at pages 399 - 410 of "Plasmids of Medical, Environmental and Commercial Importance", Editors K N Timmis and A Puhler, Published Elsevier, North Holland, 1979) and R 68.45. Included in the Inc W group are plasmids R388 (described by N Datta et al, J. Gen. Microbiol., 72, 349, (1972)) and R7K.

NB. Plasmid pACYC 184 is described by A C Y Chang et al, J. Bacteriol., 134, 1141 - 56, (1978).

We have found that the gdh gene of E. coli can be carried on a small easily manipulated DNA fragment having at its ends target sites for the SalI restriction enzyme. This enables the fragment to be conveniently transferred from one plasmid containing it to another by cutting the DNAs of the fragments with SalI. The cut DNAs are then mixed and treated with a ligase. For such a step the receiving plasmid requires a SalI target either as a normal

genotypic feature of the plasmid or imparted to it by an associated transposon or phage.

In one embodiment of the invention a gene, e.g. the gdh gene is transferred into a recipient microorganism using a multicopy number plasmid. A multicopy number plasmid is one which, when introduced into a microorganism is capable of replicating itself a significant number of times suitably to produce substantially more than 3 copies of itself per cell, often 8 or 10, preferably at least 20 copies per cell and particularly 20 to 30 copies per cell. By contrast a "single copy" number plasmid is one which replicates itself in a controlled way such that it resides in the cell as 1 to 3 copies. Using a multicopy plasmid a gene such as the gdh gene may be introduced into a cell more intensively i.e. the replication of the plasmid produces a substantial number of copies of the gene carrying DNA within the cell. Specifically in the instance of the gdh gene introduced into a methanol-utilizing bacterium such as Methylophilus methylotrophus it increases the amount of GDH enzyme in the microorganism cells and facilitates establishment therein of an efficient pathway for ammonia assimilation by the GDH pathway. The use of multicopy plasmids has important consequences. A preferred multicopy plasmid for transferring gdh gene into Methylophilus methylotrophus is R300B.

Preferably when a microorganism is modified by introduction of the gdh gene thereby enabling it to assimilate ammonia by the GDH pathway, it is also modified to prevent it using the alternative GS/GUS pathway thereby enabling it to make the most efficient use of the GDH pathway. The GS/GUS pathway can be blocked by ensuring that the microorganism lacks the glt gene.

Microorganisms lacking the glt gene can be obtained by forming mutants of the original strain e.g. of Methylophilus methylotrophus and selecting mutants which have lost this gene. Two methods of selection by (a) temperature and (b) ammonia growth are described in Example 1. Mutants can be produced by standard techniques, for example by treatment with a physical mutagen such

as gamma, X or ultra-violet radiation or a chemical mutagen such as N-methyl-N^1-nitro-N-nitroso guanidine, nitrous acid, a methane-sulphonate ester (e.g. methyl or ethyl), 5-bromo-uridine, 5-bromo-uracil, 2-amino-purine or nitrogen mustard or a biological mutagen such as Mu-phage. Treatment to produce a deletion mutant is preferred. The mutant normally remains capable of the GS catalysed reaction.

The invention is illustrated by the following examples:-

EXAMPLE 1

Transfer of E. coli gdh gene into Methylophilus methylotrophus strain

The E. coli gdh gene was transferred into Methylophilus methylotrophus strain NCIB No. 10515 by a method comprising the five steps described below.

1. Development of vector

To generate derivatives of plasmid RP4 carrying the E. coli gdh gene the following E. coli strains were constructed:-

E401 - donor strain for gdh gene, with the relevant genotypic features:

glt B, gdh$^+$, (Mu cts):RP4

E412 - recipient strain for mating with E401, derived from E. coli strain PA340 (alternativly known as CB100), with the relevant genotypic features:

glt B, gdh, rif^r, (Mu cts), rec A.

The glt B and gdh mutations render E412 glutamate dependent and in a mating with E401 only gdh gene acquistion will give glutamate independence. The rec A mutation renders E 412 recombination deficient.

The E401 cells were grown overnight at 37°C to induce Mu cts mediated mobilisation of segments of the E 401 chromosome to plasmid RP4. Cells from this overnight culture were then mated with E412 cells grown at 30°C. Matings were performed non-selectively by overnight growth at 30°C on L agar plates. Dilutions of cells recovered from the mating plates and control plates (on which donor and recipient cells had been grown separately) were then plated out and selections made for glutamate independence

(i.e. the ability to grow using ammonia as nitrogen source), rifampicin (*rif*^{r}) (a selection for recipient based cells) and kanamycin resistance (*Km*^{r}) (a marker borne by the RP4 plasmid).

The cells thus selected were E412 cells with the relevant genotypic features:

glt B, *gdh*$^+$ (Mu *cts*):RP4; i.e. cells containing an RP4 plasmid with Mu phage and *gdh* gene attached - RP4 prime *gdh* (RP4'*gdh*).

2. Transfer of *gdh* gene to small plasmid

Plasmid RP4'*gdh* is a large plasmid carrying much unknown DNA and giving problems on transfer and with DNA purification. The *gdh* gene was therefore transferred as follows to the smaller plasmid pACYC184. pACYC184 was selected because (a) it contains a gene specifying chloramphenicol resistance (*Cm*^{r})- a marker lacking in RP4- and, (b) it has targets for the restriction enzymes *HindIII*, *BamHI* and *SalI* located within the DNA of the tetracycline resistance gene (*Tc*^{r}) and therefore insertion of "foreign" DNA into these restriction sites on plasmid pACYC184 will inactivate the *Tc*^{r} gene.

The DNA of plasmid RP4'*gdh* and the DNA of plasmid pACYC184 were both cut with *SalI* restriction enzyme. The two cut DNAs were mixed and a DNA ligase was added. The DNA thus produced was introduced by transformation into *E. coli* strain PA340 (relevant genotype features: *gltB*, *gdh*). A selection was then made for cells exhibiting glutamate independence, chloramphenicol resistance (*Cm*^{r}) and tetracycline sensitivity (*Tc*^{s}). Such cells contain pACYC184 plasmids whose *Tc*^{r} genes have been cut and fragments of RP4'*gdh* DNA inserted thereinto. It should be noted that pACYC184 is a multicopy plasmid and that when inserted into a cell it will replicate to produce numerous copies. Hence cultures of selected cells produced cells containing numerous copies of the *gdh* gene.

3. Transfer of *gdh* gene to plasmid transferable to NCIB No. 10515

The plasmid pACYC184/*gdh* is not directly transferable into strain NCIB No. 10515. In this plasmid the DNA fragment containing *gdh* is located between targets for restriction enzyme *SalI*. *gdh* fragments were transferred to two alternative plasmids which can

be transferred to strain NCIB No. 10515 and which will replicate therein. These plasmids were:-

pRP301 - a single copy P group plasmid which can transfer itself into strain NCIB No. 10515, and

R300B - a multicopy plasmid which can be mobilised into strain NCIB No. 10515 by use of plasmid RP4.

Transposon Tn 5 was transposed into plasmid R300B to give pTB70 as it was required (a) to provide a SalI restriction enzyme target on this plasmid which does not have such a target (Molec. Gen. Genet., 171, 7 - 13, 1979) and (b) to provide a kanamycin resistance (Km^r) for the plasmid.

The pACYC184 gdh plasmid DNA was cut with SalI restriction enzyme and, in separate experiments, was mixed with pRP301 and pTB70 plasmid DNA similarly cut with SalI restriction enzyme. In both experiments the resulting DNA mixtures were treated with DNA ligase. The DNAs thus produced in the two experiments were separately introduced by transformation into E. coli strain PA340 (relevant genotypic features: gltB, gdh). Selections were then made in the case of pTB70 for cells exhibiting glutamate independence and kanamycin resistance (Km^r). These strains were then screened for chloramphenicol sensitivity (Cm^s). In the case of pRP301 selections were made for cells exhibiting glutamate independence and ampicillin resistance (amp) and these strains were screened for tetracycline sensitivity.

4. Transfer of gdh gene into strain NCIB No. 10515

In step 3 were produced two alternative modified E. coli strains containing respectively plasmid pRP301/gdh and plasmid pTB70:gdh. In separate experiments these two strains were used as alternative means to transfer the gdh into cells of the strain NCIB No. 10515.

Transfer using pTB70:gdh

Plasmid R300B cannot mobilise itself into cells of strain NCIB No. 10515, i.e. R300B is not transferred from a strain carrying R300B to another strain. This difficulty was overcome by inserting into the E. coli cells containing pTB70:gdh an additional

plasmid which can mobilise itself into cells of NCIB No. 10515. The extra plasmid inserted was RP4 whose properties enable it to provide a method for the transfer of plasmids such as plasmid pTB70:*gdh* from one cell to another. *E. coli* cells were therefore prepared containing the plasmids pTB70:*gdh* and RP4. A streptomycin resistance marker (*Sm*^{r}) is carried by plasmid R300B. NCIB No. 10515 lacks this resistance. This streptomycin resistance was then used to select for transfer of pTB70:*gdh* to NCIB 10515.

A culture medium suitable for strain NCIB No. 10515 but including streptomycin was prepared and placed upon a petri dish which was divided, one half being used as a control whilst cells of NCIB No. 10515 were placed on the other half. Both parts were streaked with a culture containing *E. coli* cells modified as described above. On the control part of the dish *E. coli* cells did not survive. On the other side, whilst the *E. coli* cells did not survive. it was possible for DNA material to be transmitted from them to cells of NCIB No. 10515. This enabled NCIB No. 10515 cells which had received DNA containing pTB70:*gdh* with associated (*Sm*^{r}) to survive, i.e. selection was made for (*Sm*^{r}).

The resulting bacterial strain - Modification 2 - was a modification of NCIB No. 10515 with the relevant genotypic features: *glt*$^+$, *gdh*$^+$, pTB70, *Sm*^{r}.

Transfer using pRP301/*gdh*

This plasmid was also transferred into strain NCIB No. 10515 using suitable methods to give Modification 1 - a modification of NCIB No. 10515 with the relevant genotypic features: *glt*$^+$, *gdh*$^+$, pRP301.

Thus both Modifications 1 and 2 possess both of the alternative pathways for ammonia assimilation.

5. Transfer of *gdh* gene into *glt* NCIB No. 10515

To obtain an NCIB No. 10515 strain relying entirely upon the GDH pathway for ammonia assimilation it was necessary first to obtain mutants of NCIB No. 10515 lacking the *glt* gene. Two methods were employed.

(a) Temperature sensitive mutants - NCIB No. 10515 cells were treated

with N-methyl-N^1-nitro-N-nitroso guanidine and the resulting variety of mutants was examined at 30°C and 37°C. A total of 550 temperature sensitive mutants, i.e. ones capable of growth at 30°C but not at 37°C, were collected. These were examined to find mutants in which the glt gene product had become temperature sensitive. The mutants were mated as described in 4 above with E. coli cells containing plasmids pTB70:gdh and RP4. Four mutants were, after this mating, capable of growth at 37°C. This implied that in these four cases the mutant cells were ones in which the glt gene product had become temperature sensitive but that, having acquired pTB70:gdh from the E. coli cells, growth at 37°C became possible. This result was confirmed by incorporating into separate samples of each of the original mutants pTB70:gdh and pTB70 (using the method of 4 above) and showing that only transfer of the former plasmid allowed growth at 37°C, and thus that growth was due to a function coded for on the cloned fragment of E. coli DNA carrying the gdh gene. Furthermore, these temperature sensitive mutants carrying pTB70:gdh were unable to form colonies at 37°C on plates supplemented with concentrations of ammonia at 0.07 mM or less, on which NCIB No. 10515 can form colonies. This implies that these cells are using the gdh gene product for growth since cells using the glt gene product can grow on much lower concentrations of ammonia than cells using gdh. Finally it was confirmed by enzyme assays on cells grown at 37°C that these temperature sensitive mutants carrying pTB70:gdh lacked detectable GUS activity, but had GDH activity, whereas NCIB No. 10515 had GUS activity but lacked detectable GDH activity.

One of the four temperature sensitive mutants modified by inclusion of plasmid pTB70:gdh was deposited at NCIB as NCIB No. 11585 (Modification 3) on 5 March 1980.

Further modifications were prepared by incorporating into temperature sensitive mutants deficient in the glt gene product, the plasmid pRP301:gdh.

(b) Ammonia growth Selected mutants - In separate experiments cells of Modifications 1 and 2 were used as "wild-type" strains.

These cells contain both the gdh and the glt genes. However mutants may occur lacking the glt gene. For efficient assimilation of ammonia by the GDH pathway a high concentration of ammonia is desirable. The alternative GS/GUS pathway functions well at low ammonia concentrations. Hence cells of Modifications 1 and 2, having both pathways should function well at both high and low concentrations of ammonia. In the experiments therefore cells carrying the pTB70:gdh plasmid were grown on media containing (a) >30 mM ammonia and (b) <.07 mM and cells were selected which were unable to grow on the medium containing <.07 mM. In this way mutants of strain NCIB No. 10515 were obtained which were gdh^+ and glt (some derived from Modification 1 and some from Modification 2).

EXAMPLE 2

Production of SCP using Modification 3 (NCIB No. 11585).

The organisms designated NCIB No. 10515 and NCIB No. 11585, the latter using GDH activity rather than GUS/GS, were grown in continuous culture in a methanol/mineral salts medium designed to support 30 gms/l dry weight of cells growing at a dilution rate of 0.2 hr^{-1}. Temperature was controlled at 37°C ± 1°, pH 6.8 - 7.0 by the demand addition of ammonia gas. The cultures were supplied with air as oxygen source. Modification 3 cultures were grown in the presence of not less than 50 mM ammonia in order to saturate GDH.

Carbon conversions of methanol carbon to organic cellular carbon were determined by total carbon analysis. Results gave a growth yield improvement of 4 to 7% for NCIB No. 11585 over NCIB No. 10515.

PA/JNA/MP
3 February 1981

1. A method for making a genetically modified microorganism by incorporating DNA material comprising a gdh gene into a microorganism deficient in this gene, so that expression of the gdh gene product, i.e. the active GDH enzyme, occurs in the previously gdh gene-deficient microorganism, which method comprises the steps of (a) introducing into a first microorganism having the gdh gene a plasmid vector for said gene, and (b) transferring said gene on said vector into a second microorganism.

2. A method for making a genetically modified microorganism by transferring DNA material comprising one or more genes from a donor microorganism having said gene or genes to a recipient microorganism deficient therein wherein the DNA material is transferred into the recipient microorganism on a plasmid vector formed from a multicopy plasmid.

3. A method for making a genetically modified microorganism by transferring DNA material comprising the gdh gene from a donor microorganism having said gene to a recipient microorganism deficient therein which comprises a step wherein the gdh gene containing DNA material is transferred on a DNA fragment having at its ends target sites for the SalI restriction enzyme.

4. A method according to claim 1 wherein the second microorganism is an intermediate and the gdh gene is transferred via one or more intermediate microorganisms from the first microorganism to the gdh gene-deficient microorganism.

5. A method according to claim 2 which comprises a plurality of steps involving at least one intermediary microorganism during transfer of the DNA material from the donor to the recipient microorganism.

6. A method according to claim 2 or claim 5 wherein the gene transferred is a gdh gene.

7. A method according to claim 3 wherein the DNA fragment transferred is transferred on a plasmid vector formed from a multicopy plasmid.

8. A method according to any one of the preceding claims wherein a gdh gene is transferred into a gram-negative bacterium

lacking this gene.

9. A method according to claim 8 wherein the gram-negative bacterium is a strain of the species Methylophilus methylotrophus.

10. A method according to claim 9 wherein the strain is Methylophilus methylotrophus NCIB No. 10515.

11. A method according to any one of the preceding claims which comprises a step in which the glt gene product is inactivated by mutation in gdh gene-deficient microorganism.

12. A method according to any one of the preceding claims wherein the gdh gene containing DNA material is obtained from a strain of E. coli.

13. A method according to any one of the preceding claims wherein the gdh gene containing DNA material is transferred into the gdh gene deficient microorganism on a plasmid vector formed from an Inc Q, Inc P or Inc W group plasmid.

14. A method for making a genetically modified microorganism substantially as described and as shown in Example 1.

15. Genetically modified microorganisms produced by a method according to any one of the preceding claims and variants and mutants of such genetically modified microorganisms.

16. Genetically modified microorganisms according to claim 15 which are genetically modified strains of gram-negative bacteria.

17. Genetically modified microorganisms according to claim 16 which are strains of the species Methylophilus methylotrophus genetically modified by incorporation of DNA material comprising a gdh gene.

18. The microorganism Methylophilus methylotrophus strain NCIB No. 11585 and variants and mutants thereof.

19 A process for the production of single cell protein and/or an amino acid wherein a gram-negative bacterium genetically modified by incorporation of DNA material comprising a gdh gene and produced by a method according to any one of claims 1 to 14 is aerobically cultured in an aqueous medium containing methanol as a source of assimilable carbon and ammonia and/or ammonium ion at a concentration sufficient to maintain the GDH reaction

mechanism and the single cell protein, a composition containing it and/or an amino acid is recovered from the culture medium.

20. A process according to claim 19 wherein the genetically modified microorganism is a genetically modified strain of the species Methylophilus methylotrophus.

21. A process according to claim 20 wherein the genetically modified microorganism is Methylophilus methylotrophus strain NCIB No. 11585 or a variant or mutant thereof.

22. Dead cells of microorganisms according to any one of claims 15 to 17.

23. Protein derived from microorganisms according to any one of claims 15 to 17.

24. A foodstuff for animal or human consumption containing dead cells according to claim 22 or protein according to claim 23.

25. Modified plasmids produced at intermediate stages of a method according to any one of claims 1 to 14.

26. Modified R300B, pRP301, pACYC184 plasmids incorporating the gdh gene.